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SUMMARY. This paper expands on a previous report about coronaviruses in quail. After surveillance carried out in 2009 and 2010, some farmers started vaccinating quail with the Massachusetts avian infectious bronchitis virus serotype. The samples for this study were collected in 2013 from São Paulo state in southeastern Brazil. Pools of trachea, lungs, reproductive tract, kidneys, and enteric contents from quail and laying hens kept in the same farms and from quail-only farms as well as from both healthy birds and those showing infectious bronchitis–like symptoms were sampled in this study. The samples were screened using nested RT-PCR targeting the 3′-untranslated region of the Gammacoronavirus genus. Based on the DNA sequence for the RNA-dependent RNA polymerase (RdRp) gene, the strains isolated from quail clustered within either the Gammacoronavirus or Deltacoronavirus genus, and sequences from both genera were found in one quail sample. The phylogeny based on the partial S1 subunit sequence showed that the gammacoronaviruses detected in quail and layers belonged to the Brazil type. These results suggest that quail are susceptible to Gammacoronavirus and Deltacoronavirus viruses and indicate that the Massachusetts vaccination was not controlling IBV in quail or chickens.

RESUMEN. Gammacoronavirus y Deltacoronavirus en codornices.

En este trabajo profundiza un reporte anterior sobre coronavirus en codornices. Después de la vigilancia llevada a cabo en los años 2009 y 2010, algunos productores comenzaron a vacunar codornices con el virus de la bronquitis infecciosa serotipo Massachusetts. Las muestras para este estudio fueron recolectadas en el año 2013 del estado de Sao Paulo, en el sureste de Brasil. En este estudio se recolectaron muestras agrupadas de tráquea, pulmones, tracto reproductivo, riñones y contenidos intestinales de codornices y de gallinas postura alojadas en la misma granja y de granjas exclusivas de codornices, así como de aves sanas y de aquellas con signos clínicos similares a los de la bronquitis infecciosa. Las muestras fueron seleccionadas utilizando un método RT-PCR anidado dirigidos a la región 3′ no traducida del género Gammacoronavirus. Con base en la secuencia de ADN para el gene de la polimerasa de ARN dependiente de ARN (RdRp), las cepas aisladas de codorniz se agruparon ya sea dentro de los géneros Gammacoronavirus o Deltacoronavirus y secuencias de ambos géneros fueron encontrados en una muestra de codorniz. La filogenia basada en la secuencia parcial de la subunidad S1 mostró que los gammacoronaviruses detectados en codornices y en gallinas de postura pertenecían al tipo de Brasil. Estos resultados sugieren que las codornices son susceptibles a los virus Gammacoronavirus y Deltacoronavirus e indican que la vacunación con serotipo Massachusetts no estaba controlando al virus de la bronquitis infecciosa en codornices o en pollos.

Key words: coronaviruses, quail, laying hens, IBV, Gammacoronavirus, Deltacoronavirus

Abbreviations: aMPV = avian metapneumovirus; DEPC = diethyl pyrocarbonate; EC = enteric contents; FRT = female reproductive tract; IB = infectious bronchitis; IBV = avian infectious bronchitis virus; NDV = Newcastle disease virus; Phred = Phil's Read Editor; QCoV = quail coronavirus; RdRp = RNA-dependent RNA polymerase; RT-PCR = reverse transcription–polymerase chain reaction; S gene = spike gene; SPF = specific-pathogen-free; T = trachea; UTR = untranslated region; v/v = volume/volume

Surveillance was carried out in 2009 and 2010 in Brazil by Torres et al. (25) for avian coronavirus (avian infectious bronchitis virus [IBV]), and IBV was detected in quail and layers with and without clinical signs compatible with infectious bronchitis (IB). After this surveillance, some quail farmers began to vaccinate quail against IBV with live attenuated or inactivated vaccines based on the Massachusetts (Mass) serotype. Nevertheless, IB outbreaks continued to occur in vaccinated quail flocks and were caused by Brazilian field strains of a serotype divergent from the vaccine strain, as previously report in chickens (1,2,10,15,23,24,28,29). Avian coronavirus is a Gammacoronavirus that can replicate on most epithelial surfaces, causing an economically significant poultry disease that affects renal, reproductive, and respiratory systems and causes enteritis (5,6,21,28). Other respiratory pathogens, such as the

Newcastle disease virus (NDV) and avian metapneumovirus (aMPV), can interact with IBV increasing the severity and course of the disease (22). In addition to IBV, coronaviruses from the Gammacoronavirus (γ-Co V) and Deltacoronavirus (δ-Co V) genera can also be found in asymptomatic birds (4,11,14,15,19,20,22,30).

This investigation aimed to study the molecular diversity of coronaviruses in quail (Coturnix coturnix japonica) housed in quail-only farms and in proximity to laying hens in order to understand the viral diversity that remains a constant challenge for these host populations despite vaccination.

MATERIALS AND METHODS

Source of viruses. In 2013, samples were collected from four farms: two quail-layer farms previously sampled in 2009 and 2010 (25) were identified as Quail-layer Farm 1 and Farm 2, and two quail-only farms

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were identified as Quail Farm 3 and Farm 4. All four farms were located in the state of São Paulo, municipalities of Bastos and Iacri, in southeastern Brazil. This area was chosen because it is a high-density poultry region and one of the most important for egg production. The quail and chicken flocks exhibited disorders of the respiratory and reproductive tracts including the following symptoms: nasal discharge, gasping, watery eyes, conjunctivitis, and a drop in egg production as well as eggs with thin-walled and misshapen shells and loss of pigmentation.

The chickens used in this study had been vaccinated against IBV using attenuated and inactivated Massachusetts vaccines, including aMPV with subtype A or B, and against NDV with a lentogenic vaccine. The quail used in this study were vaccinated on only two farms against IBV: one of them (Quail Farm 4) vaccinated their quail within the first 2 months of age using an attenuated plus inactivated Massachusetts vaccine, and the other (Quail-layer Farm 1) vaccinated their quail with only an attenuated Massachusetts vaccine. All quail were vaccinated with the lentogenic NDV vaccine but not with the vaccine against aMPV.

Samples from 18 flocks (12 quail flocks and 6 chicken flocks) were collected as pools of the female reproductive tract (FRT), lungs, kidneys, trachea (T), and complete enteric contents (EC) from five birds (quail or chicken) per house with and without clinical signs of viruses for a total of 90 samples.

RT-PCR controls. The IBV Massachusetts strain vaccine (Nobilis™ IB Ma5; MSD Animal Health, Boxmeer, the Netherlands), aMPV vaccine RTV 8544 (Nobilis RTV 8544; MSD Animal Health) and lentogenic NDV vaccine (ND LA Sotal Nobilis; MSD Animal Health) were used as positive controls for reverse transcription–polymerase chain reaction (RT-PCR). Ultra-pure water treated with 0.1% diethyl pyrocarbonate (DEPC) was included as a negative control.

RNA extraction. A pool of each organ and enteric contents was prepared as a 50% (v/v) suspension in DEPC-treated water, subjected to three freeze-thaw cycles in liquid nitrogen and at 56 C, and then clarified by centrifugation at $5000 \times g$ and 4 C for 15 min. The total RNA was extracted from the supernatants using TRIzol Reagent™ (Life Technologies, Carlsbad, CA), according to the manufacturer's instructions.

Synthesis of complementary DNA (cDNA). Each RNA sample (3.5 µl) was denatured at 94 C for 5 min and reverse-transcribed using Random Primers™ and M-MLV Reverse Transcriptase™ (Life Technologies) following the manufacturer's instructions. For the partial amplification of the pan-coronavirus RNA-dependent RNA polymerase (*RdRp*) gene and Spike (S) gene of IBV (positive samples only), the reverse transcription steps were carried out using specific primers and ThermoScript™ Reverse Transcriptase (Life Technologies).

Avian coronavirus, aMPV, and NDV screening. Each sample was screened for IBV, aMPV, and NDV as previously described (9,8,27), targeting the 3′-untranslated region (UTR) and G and F genes, respectively. GoTaq™ Green Master Mix (Promega, Madison, WI) was used following the manufacturer's instructions to generate amplicons of 179, 268, or 361 (aMPV types A or B) and 255 bp, respectively.

RNA-dependent RNA polymerase (*RdRp*) and Spike-gene sequence analysis. The 3′ UTRs of avian coronavirus positive samples were then tested using pan-coronavirus RT-PCR targeting the *RdRp* gene (440 bp amplicon) as previously described (11). A typing S-gene targeted multiplex RT-PCR for Mass D274 and 4/91 genotypes (8) and a pan-IBV S1 gene (avian coronavirus) with primers Braco forward ACTCTTTTGTGTGCACTAT and Braco reverse CTTTGGTCTCATTAGAAGTAAAAAC PCR and Braco2 reverse TAGYYACASDAACTYTTAAATTATA for hemi-nested PCR (450 bp amplicon). The amplicons were purified from agarose gels using the GFX PCR DNA and Gel Band Purification Kit™ (GE Healthcare, Amersham, UK), and the concentration was visually assessed using a Low DNA Mass Ladder™ (Life Technologies) according to the manufacturer's instructions. The samples were subjected to bidirectional DNA sequencing using Big Dye™ 3.1 (Cycle Sequencing Kit,

Applied Biosystems, Carlsbad, CA) and an ABI-3500 Genetic Analyzer (Applied Biosystems).

All chromatograms were manually checked using Finch TV program 1.4.0 (© 2004–2006 Geospiza Inc.) and were submitted to quality evaluation using the Phil's Read Editor (Phred) online application (available in http://asparagin.cenargen.embrapa.br/phph/) with a base-quality cutoff of 20. The final sequence of each sample was obtained with the Cap-Contig application included in Bioedit 7.0.9.0 software (17) and aligned with homologous sequences retrieved from GenBank (accession numbers are shown in Figs. 1 and 2). Nucleotide (Neighbor-joining Maximum Composite Likelihood model, 1000 bootstrap replications) trees for the S gene and *RdRp* were assembled using MEGA 5.2.1 (26).

Cloning of PCR products. To assign dubious chromatogram peaks in *RdRp* amplicons of two quail samples from Quail-layer Farm 2 and Quail Farm 3, PCR products were cloned prior to DNA sequencing as described above. Briefly, 440 bp amplicons were purified using the Illustra GFX PCR DNA and Gel Band Kit™ (GE Healthcare) and individually inserted into pGEM™-5Zf (+) plasmids (pGEM™-T Vector System II™, Promega), according to the manufacturer's instructions. These plasmids were then used to transform *Escherichia coli* strain JM109 competent cells. The presence of inserted amplicons in each clone was checked by PCR using GoTaq® Green Master Mix (Promega) and M13/pUC primers following the manufacturer's instructions.

Virus isolation. Four quail coronavirus positive pools (kidneys, enteric contents, reproductive tract, and lungs + trachea) negative for aMPV and NDV, one from each farm, were used for virus isolation in SPF eggs (16). On day 3 post-inoculation, three eggs per sample were removed from the incubator and were incubated at 4 C for 18 hr. The chorioallantoic fluids of the inoculated eggs were harvested and stored at −80 C until RNA extraction could be performed using the PureLink™ RNA Mini Kit (Ambion, Carlsbad, CA). The remaining eggs were incubated up to 7 days and observed for typical IBV lesions. Four blind serial passages were performed in the same way to complete five blind passages per sample. Virus isolation was monitored by screening with RT-PCR for all viruses described above using the collected allantoic fluids as samples. PBS was included as a negative control.

Statistical analysis. The association between the species of bird (quail and laying hens) and the presence of virus was determined with Fisher's exact test using Minitab 15.1.0.0 (© 2006 Minitab). The frequency of the occurrence of coronavirus in quail and laying hens was determined using the ratio between the number of positive samples from the hemi-nested RT-PCR for the 3′ UTR for each class of samples and all samples tested in each class.

RESULTS AND DISCUSSION

In this investigation, the presence of *Gammacoronavirus* was identified for all the farms in this study. As previously reported (25), an avian coronavirus closely related to an infectious bronchitis virus was identified in the studied area. At this time, 88% (16/18) of the flocks were considered positive, including the simultaneous detection of the virus in quail (46.6%; 28/60) and chicken (20%; 6/30) pools from birds both with and without clinical signs compatible with infectious bronchitis. The results per bird and sample types are shown in Table 1. Moreover, there was no statistically significant difference in the *Gammacoronavirus* frequency between layers and quail using Fisher's exact test ($P = 1$). aMPV subtype B was detected in only one pool of the reproductive tract of chickens, and NDV was not detected in any sample.

From the 18 amplicons from quail and chickens (between the *RdRp* and the S genes), eight sequences of sufficient quality (Phred
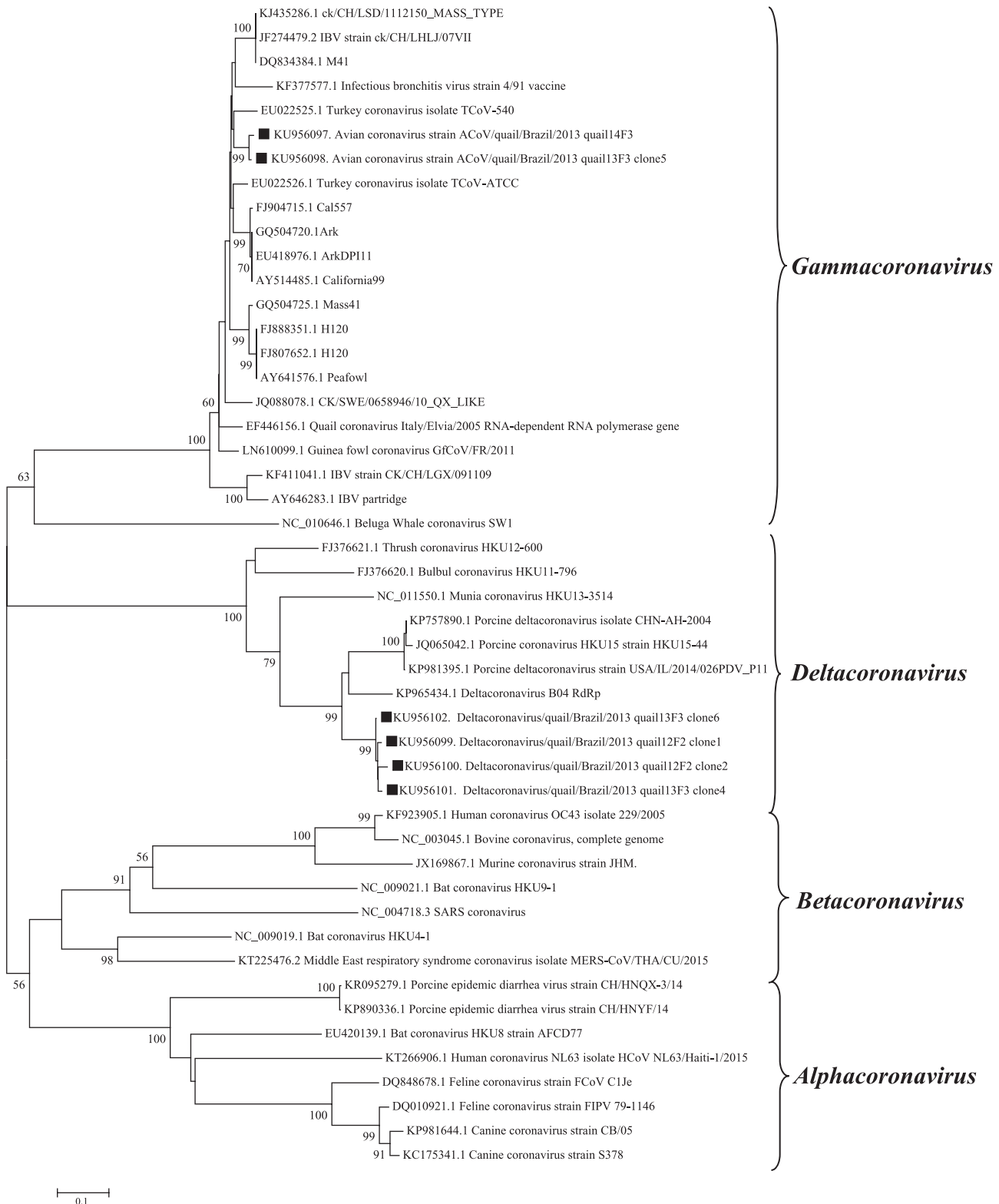
Fig. 1.   Phylogenetic distance tree using the Neighbor-Joining algorithm and MCL method for nucleotides of the *RdRp gene* (nt positions 16,179–16,543), showing the classic serotypes/genotypes (with GenBank accession numbers) of the genera *Alphacoronavirus, Betacoronavirus, Gammacoronavirus,* and *Deltacoronavirus* and strains included in this study (square represents quail). Numbers above each node represent the bootstrap values for 1000 replicates (only values greater than 50% are shown). The bar represents the number of nucleotide substitutions per site.
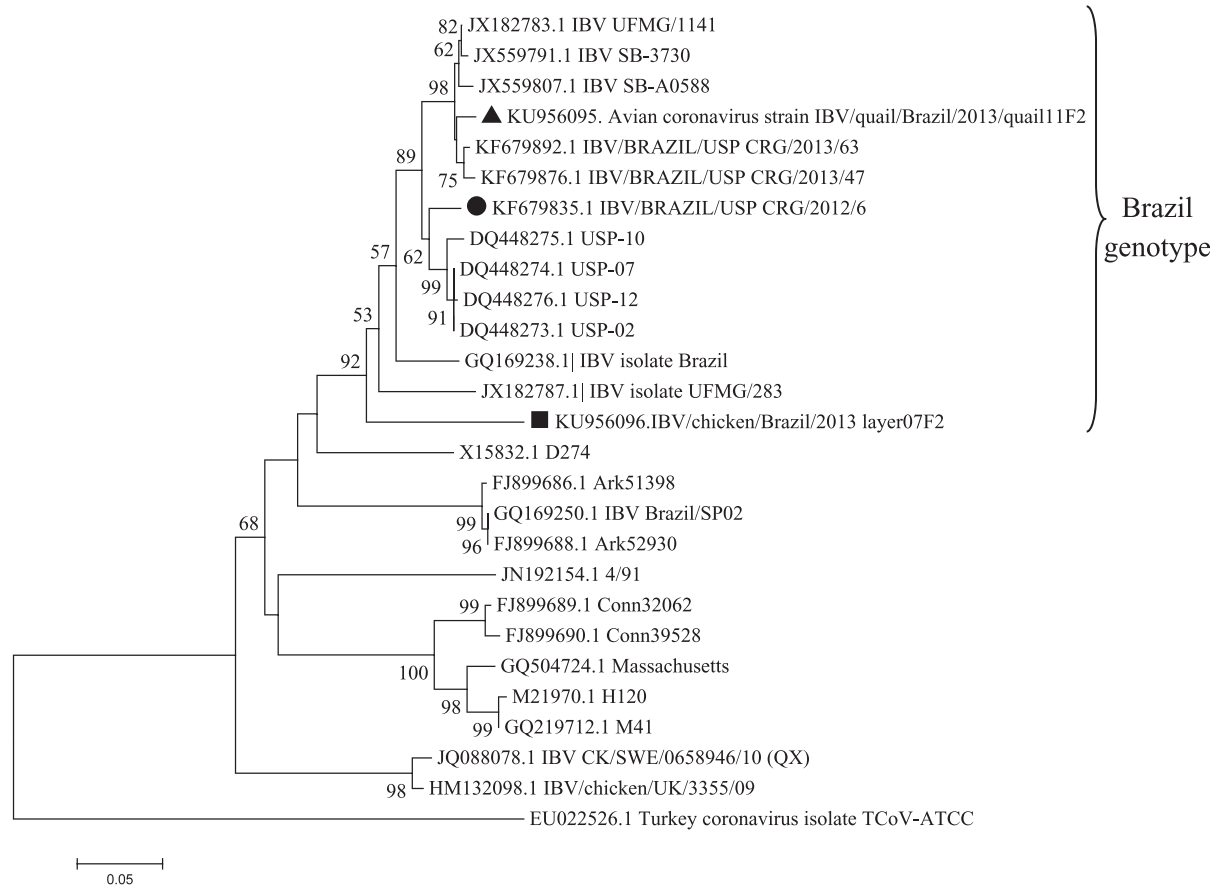
Fig. 2.   Phylogenetic distance tree using the Neighbor-Joining algorithm and MCL method for nucleotides of the S gene S1 subunit (nt positions 28–477) showing classic serotypes/genotypes (with the accession number from GenBank) and sequences from this study (square represents a strain from a layer hen, the triangle a quail strain and the circle the CRG strain from a poultry flock previously isolated in the laboratory from SPF eggs). The numbers above each node represent the bootstrap values for 1000 replicates (only values greater than 50% are shown). The bar represents the number of nucleotide substitutions per site.

score > 20) were obtained, as follows: six *RdRp* PCR and cloning products from the enteric contents (Farm 3), female reproductive tract (Farms 2 and 3) and trachea (Farm 2) pools of quail, and two hemi-nested PCR products targeting the S1 gene from quail and layers (housed on the same property) EC (Table 2).

The *RdRp* tree (Fig. 1) indicated that two quail strains (Farm 3), one from the original sample of enteric contents (Quail14/EC/Farm3) and the other from the cloning product of the female reproductive tract (Quail13/FRTclone5/Farm3), clustered with other gammacoronaviruses, whereas four clones, two from the FRT of Farm 3 (Quail13/FRTclone4/Farm3 and Quail13/FRTclone5/Farm3) and two from the T of Farm 2 (Quail12/Tclone1/Farm2 and Quail12/Tclone2/Farm2), were clustered with other deltacoronaviruses, showing that quail are susceptible to both coronavirus genera.

Table 1.   Number of positive results/number of samples tested using RT-PCR targeting the 3′ UTR of *Gammacoronavirus* in quail and chickens.

	Kidneys	Reproductive tract	Lungs	Trachea	Enteric contents	Total
Quail	5/12	5/12	1/12	7/12	10/12	28/60
Chicken	1/6	0/6	0/6	0/6	5/6	6/30
Total	6/18	5/18	1/18	7/18	15/18	34/90

The mean *RdRp* nucleotide identities of the quail strains Quail14/EC/Farm3 and Quail13/FRTclone5/Farm3 were 94% similar to the other gammacoronaviruses, and the strains Quail13/FRTclone4/Farm3, Quail13/FRTclone5/Farm6, Quail12/Tclone1/Farm2, and Quail12/Tclone2/Farm2 were 87% similar to other *Deltacoronavirus*.

The analysis of the S1 subunit (Fig. 2) revealed the circulation of two lineages in the Brazilian type of *avian coronavirus* in the same farm (Quail-Layer Farm 2; one in quail and one in chickens), in agreement with the fact that IBV types other than the archetypical ones are widespread in Brazilian poultry farms (2,10,13,29). Moreover, genotyping results showed that all the gammacoronaviruses detected in samples from quail and layers were different from the Mass genotypes D274 and 4/91.

In areas where there are many poultry farms, as in this case, it is virtually impossible to keep chickens free of IBV. As the virus readily spreads, vaccination is commonly practiced (7,18). Despite the introduction of a vaccination program in some farms using the only serotype permitted in Brazil, which is a live attenuated vaccine (Massachusetts), *Gammacoronavirus* continued to be reported in quail as well as in chickens (2,10,29).

The simultaneous detection of *Gammacoronavirus* and *Deltacoronavirus* in the same flock (Quail Farm 3) indicates that these viruses might have co-circulated in the studied farm. The co-infection with

Table 2. Strains included in the analysis of the RNA-dependent RNA polymerase (*RdRp*) and S genes according to clinical signs at the time of sampling (2013) and type of sample.

Strain	Signs	Sample	GenBank accession number
Quail12/Tclone1/F[A]2	Rep[C]	Trachea	KU956099
Quail12/Tclone2/F2	Rep	Trachea	KU956100
Quail13/FRTclone4/F3	Res[B] Rep	Female reproductive tract	KU956101
Quail13/FRTclone5/F3	Res Rep	Female reproductive tract	KU956098
Quail13/FRTclone6/F3	Res Rep	Female reproductive tract	KU956102
Quail14/EC/F3	Res Rep	Enteric content	KU956097
Quail11/EC/F2	Rep	Enteric content	KU956095
Layer7/EC/F2	Res	Enteric content	KU956096

[A]Farm.
[B]Respiratory.
[C]Reproductive.

two or more coronaviruses in different birds within a region might not be unexpected because this phenomenon has been observed previously (3) with IBV in chickens and co-infection with both *Gammacoronavirus* and *Deltacoronavirus* was reported in ducks (11).

Further efforts to sequence the entire genome of the quail coronaviruses detected herein using next generation sequencing (NGS) will be carried out by the authors to better elucidate the origin of the *Deltacoronavirus RdRp* genes. This will provide valuable insight into the mechanisms behind the coronavirus evolutionary biology and subsequently contribute to better control measures to prevent vaccine failures (18).

Although the *Gammacoronavirus* strains detected in this study could not be isolated in embryonated chicken eggs, the sequence homology analysis of the sequenced PCR products obtained both in the replicase region and in the S1 subunit were evidence that viral RNA was present in the surveyed birds (12).

In vivo pathogenicity assays using a quail Avian coronavirus still need to be carried out, but the results presented herein provide advances over previous studies of quail coronaviruses as follows: (a) quail avian coronaviruses share spike genes identical to chicken IBV spike genes; (b) vaccination against IBV with the Massachusetts strain does not provide sufficient control of IB in quail when Brazilian type field strains are involved, similarly to what happens in chickens; and (c) quail are susceptible to both *Gammacoronavirus* and *Deltacoronavirus*.

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ACKNOWLEDGMENTS